THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, [illegible]7, Issue of July 5, pp. 16300–16309, 1996

Printed in U.S.A.

Antigen Binding Properties of Purified Immunoglobulin A and Reconstituted Secretory Immunoglobulin A Antibodies*

(Received for publication, January 18, 1996, and in revised form, March 13, 1996)

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The hybridoma cell line ZAC3 expresses *Vibrio cholerae* lipopolysaccharide (LPS)-specific mouse IgA molecules as a heterogeneous population of monomeric (IgA_m), dimeric (IgA_d), and polymeric (IgA_p) forms. We describe a gentle method combining ultrafiltration, ion-exchange chromatography, and size exclusion chromatography for the simultaneous and qualitative separation of the three molecular forms. Milligram quantities of purified IgA molecules were recovered allowing for direct comparison of the biological properties of the three forms. LPS binding specificity was tested after purification; IgA_d and IgA_p were found to bind strongly to LPS whereas IgA_m did not. Secretory IgA (sIgA) could be reconstituted *in vitro* by combining recombinant secretory component (rSC) and purified IgA_d or IgA_p, but not IgA_m. Surface plasmon resonance-based binding experiments using LPS monolayers indicated that purified reconstituted sIgA and IgA molecules recognize LPS with identical affinity (K_A 1.0×10^8 M^{-1}). Thus, this very sensitive assay provides the first evidence that the function of SC in sIgA complex is not to modify the affinity for the antigen. K_A falls to 6.6×10^5 M^{-1} when measured by calorimetry using detergent-solubilized LPS and IgA, suggesting that the LPS environment is critical for recognition by the antibody.

Secretory IgA (sIgA),[1] the principal immunoglobulin in mucous membrane secretions, consists of two monomeric IgA units and two additional polypeptide chains, J chain and secretory component (SC). The four constituent polypeptides are produced by two distinct types of cells. The heavy, the light, and the J chains are synthesized and assembled by plasma cells. SC corresponds to the five extracellular domains of the poly-Ig receptor and is contributed by the epithelial cells of mucous membranes and exocrine glands. During passage through the epithelium, dimeric IgA with attached J chain becomes associated with SC to form fully assembled sIgA. Despite the discovery of sIgAs in the early sixties, their mechanism of action remains poorly understood, mainly because of the difficulty in producing sufficient amounts of purified dimeric IgA or sIgA antibodies in a nonaggregated, native conformation.

IgA produced in large quantities from hybridoma cell lines can potentially be used for passive protection or therapeutic intervention on mucosal surfaces. For instance, monoclonal IgA antibodies directed against respiratory syncytial virus applied passively to the nasopharyngeal mucosa subsequently prevented initial infection and pneumonia (Weltzin *et al.*, 1994). Passive oral delivery of IgA antibodies protected also against bacterial infections in the intestine of mice (Winner *et al.*, 1991; Michetti *et al.*, 1992; Apter *et al.*, 1993; Blanchard *et al.*, 1995). However, relatively high doses of antibodies have to be applied to ensure protection, because of 1) the heterogeneity of IgA hybridoma products (as for instance monomers, single heavy, and light chains), and 2) the lower proteolytic stability of IgAs without bound SC (Mestecky *et al.*, 1991). Such a passive treatment would therefore gain in efficiency if a purification procedure of biologically active IgA dimers with SC binding capacity would be available.

IgA and sIgA have been purified on a laboratory scale from a variety of sources including milk (Brandtzaeg, 1970; Woodard *et al.*, 1984; Bouige *et al.*, 1990; Parr *et al.*, 1995), rat bile (Lemaître-Coelho *et al.*, 1977; Taylor and Dimmock, 1985), transfected mouse myelomas (Terskikh *et al.*, 1994), and transfected insect cells (Carayannopoulos *et al.*, 1994). However, these methods suffer from the difficulty of obtaining highly purified and properly separated mono-, di-, and polymeric forms in sufficient quantities to allow for direct comparison of the biological properties of these antibody molecules. The present article describes methods for the purification of different molecular forms of IgA in milligram quantities from an anti-*Vibrio cholerae* mouse hybridoma cell line. The ability of the different molecular forms to bind with antigen and with recombinant SC have been studied extensively. We demonstrate using surface plasmon resonance that SC does not modify the affinity of IgA for the antigen. The readily scalable procedure described enables the preparation of biologically active IgA and sIgA molecular forms which eventually will contribute to a fuller biochemical understanding of how the function of this important class of antibodies is mediated.

EXPERIMENTAL PROCEDURES

Cell Line and Antibody Production

The murine hybridoma cell line ZAC3 is a fusion of a lymphocyte from Peyer's patches of a BALB/c mouse orally immunized with *V. cholerae* Inaba strain with the myeloma cell line Sp/2.0. ZAC3 secretes

* This work was supported by research funds from Swiss National Science Foundation, Biotechnology Priority Program, Grants 5002-34603, 5002-38009, and 5002-35180. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: sIgA, secretory IgA; IgA, immunoglobulin A; SC, secretory component; rSC, recombinant human SC; LPS, lipopolysaccharide; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; HRP, horseradish peroxidase; IgG, immunoglobulin G.

IgA antibodies corresponding to the human allotype A2 m(1) (Mestecky and Kilian, 1985) directed against surface lipopolysaccharide (LPS). The cultures were grown in a protein-free Turbodoma FMX standard medium (F. Messi Cell Culture Technologies, Switzerland) in a 2-liter continuous stirred tank reactor (Biolafitte, France) with a working volume of 1.6 liters. The pH was maintained at pH 7.3, the dissolved oxygen concentration was automatically controlled at 80% air saturation, and the temperature was maintained at 36.5 °C. During production, the reactor was operated with a medium feed rate of 1.2 liters/day. For cell separation the harvest was filtered through a Sartoclean CA membrane capsule with pore sizes of 0.65 and 0.8 μm (Sartorius, Germany). The cell-free culture harvest containing the IgA antibodies was stored under sterile conditions at 4 °C until further required.

IgA Concentration

10-liter harvests were concentrated with a cross-flow membrane ultrafiltration system (Skan AG, Switzerland) equipped with a type Omega low protein binding membrane (100-kDa molecular mass cut-off) of surface area 2.46 m^2. Filtration was performed such that one-third of the liquid passed the membrane and two-thirds remained in the system. The solutions were 10-fold concentrated and diluted again to the starting volume with loading buffer (10 mM potassium phosphate, 100 mM NaCl, 0.02% sodium azide, pH 7.3) for subsequent diafiltration. The final retentates of 10-liter culture harvests were stored at 4 °C until further required. Protein concentration of small volumes (50–300 ml) was carried out using a 50-ml Amicon stirred cell equipped with an Omega type membrane (10 kDa cut-off value) at a pressure of 1 bar. Concentration reduced the starting volume to 5 ml.

Column Chromatography

Column chromatography was performed with a HiLoad™ system (Pharmacia Biotech Inc.) comprising an UV-M II monitor, P-50 pump, a gradient programmer GP-10, a SuperFrac fraction collector, and a recorder model 102. All steps were performed at 4 °C unless otherwise indicated. The final retentate of a 10-liter cell culture harvest was loaded onto a 500-ml DEAE-Sepharose FF (Pharmacia) column (5 cm × 25 cm) equilibrated in loading buffer. Following washes with 2 column volumes, stepwise elution was performed with 2 column volumes each of 10 mM potassium phosphate (pH 7.3) containing successively 200 mM, 300 mM, and 500 mM NaCl at a flow rate of 30 cm/h. The profile of total protein was monitored by absorbance at 280 nm, and the IgA-containing fractions were identified by ELISA. These latter were pooled, diluted twice with 10 mM potassium phosphate (pH 7.3) and passed over a 50-ml DEAE-Sepharose FF column (2.6 cm × 10 cm), washed, and eluted as above. The concentrated IgA was then separated by size-exclusion chromatography on a Sephacryl S-300 (Pharmacia) column (2.6 cm × 200 cm) equilibrated and run in PBS (Sambrook *et al.*, 1989) containing 0.02% sodium azide. Chromatography was carried out at room temperature with a flow rate of 6.1 cm/h (0.5 ml/min). Elution of total protein was monitored by absorbance at 280 nm. Fractions containing the same molecular form of IgA were pooled and diluted twice with ultrapure water prior to final concentration by DEAE-Sepharose FF chromatography in a 5-ml column (1.6 cm × 5 cm) equilibrated in loading buffer. Elution was carried out using a linear salt gradient ranging from 200 mM to 500 mM NaCl over 10 column volumes. The IgA concentration of each molecular form was determined by ELISA, and the purity was checked by SDS-PAGE and immunoblotting. The IgA solutions were stored at 4 °C. As an alternative approach, 20 ml of protein concentrate was passed over an AcA 22 Ultrogel (Biosepra, France) size-exclusion column (2.8 cm × 180 cm) equilibrated in 50 mM borate (pH 8.5), 150 mM NaCl, 0.02% NaN_3 at a flow rate of 2 cm/h (0.3 ml/min).

Anti-mouse IgA α Chain-specific ELISA

Total IgA concentration was determined by sandwich ELISA. Goat anti-mouse IgA α chain-specific antiserum (Sigma) diluted 1:500 in 50 mM bicarbonate buffer (pH 9.6) was used to coat wells (50 μl/well) of Immulon (Dynatech) 96-well plates overnight at 4 °C, which were subsequently blocked (250 μl/well) with 1% BSA (Fluka) in PBS, 0.1% Tween 20 (Sigma) at 37 °C for 30 min. Between all antibody incubation steps except the last one, the plates were washed three times with PBS, 0.01% Tween 20. IgA samples and mouse IgA standards (Sigma; range 0.4 ng-7.5 ng) were diluted in blocking buffer, and 50 μl were applied, in duplicate, to the wells. After incubation for 2 h at 37 °C, IgA was detected with biotinylated goat anti-mouse IgA antibodies (Amersham) followed by coupling with streptavidin-horseradish peroxidase (HRP) conjugate (Amersham) and development with ortho-phenyldiamine/H_2O_2. The reaction was stopped by the addition of 0.01% sodium azide in citrate buffer (pH 5.0). The absorbance was read at 492 nm and 629 nm, with the latter serving as reference reading.

LPS-specific ELISA

The antibody binding specificity was detected by sandwich ELISA as above exept that LPS (lipopolysaccharide from *V. cholerae*, serotype Inaba 569B, Sigma) was used as the capture reagent. For each assay, three ELISA plates were needed. Plate 1 was used to measure the concentration of total IgA in the purified monomer, dimer, and polymer samples. Plate 2 served to test the specificity of association of the different molecular forms of IgA to the LPS antigen. Plate 3 allowed the percentage of LPS binding activity of IgA antibody molecules to be determined indirectly.

Plates 1 and 3 were coated with 50 μl of goat anti-mouse IgA (α chain) antiserum diluted 1:500 in 50 mM sodium bicarbonate buffer (pH 9.6), while plate 2 was coated with 50 μl of 40 μg/ml LPS in the same buffer. Plates 1 and 2 were incubated for 3 h at 37 °C and blocked as above, while plate 3 was incubated overnight at 4 °C. Tetra-applicates of IgA samples and duplicates of mouse IgA standards (50 μl) in blocking buffer were applied into the wells of plates 1 and 2 and incubated overnight at 4 °C. The following day, plate 1 was developed according to the protocol given above for α chain-specific ELISA, yielding values of total IgA. Plate 3 was blocked for 30 min at 37 °C, prior to transfer of 45 μl of each IgA sample of plate 2 and 45 μl of freshly diluted IgA standards, incubated for 2 h at 37 °C, then developed as above to yield the concentration of LPS-unbound IgA. The LPS binding activity of the different IgA forms was finally calculated by subtracting the concentration values of plate 3 from the corresponding values of plate 1.

Photometric Determination of Protein Concentration

Total protein concentrations were determined using the Bradford-based protein assay (Bio-Rad). For the estimation of protein concentration, an extinction coefficient of 1.3 was used (Stoscheck, 1990; Vaerman, 1995), so that 1 unit A_{280} = 1/1.3 mg.

In Vitro Reassociation of IgA and SC

Monomeric, dimeric, and multimeric IgA from mouse hybridoma ZAC3 obtained by the purification procedure given above were combined with purified recombinant human SC (rSC; Rindisbacher *et al.* (1995)) in PBS and incubated for 16 h at ambient temperature. Molar ratios and the amount of protein used are indicated in the figure legends. Formation of covalent complexes was assayed by SDS-PAGE and Western blotting. For antigen binding experiments (ELISA, SPR), reassociated secretory IgA was prepared in the presence of a molar excess of recombinant rSC, followed by size-exclusion chromatography to separate sIgA from the excess of rSC. For protein amounts below 100 μg, the samples were passed over a 1 cm × 30 cm Superose 12 HR 10/30 column (Pharmacia) at 0.2 ml/min. For the preparation of 0.5 mg of protein and above, the samples were chromatographed on a Superdex 200 (Pharmacia) column (1.6 cm × 140 cm) run at 0.6 ml/min. Equilibration and elution were carried out in PBS. To ensure a constant flow rate, both columns were coupled to a FPLC system (Pharmacia), with continuous monitoring at 278 nm. The identity of the polypeptides in the column fractions was checked by immunoblotting with antisera against rSC and against IgA α chain. The resulting complex stoichiometry was assessed by ELISA.

Electrophoretic Methods

Protein SDS-PAGE—Gel electrophoresis of proteins was carried out in a mini-Protean II apparatus (Bio-Rad), according to the method of Laemmli (1970). PAGE was performed under nonreduced denaturing conditions (1% SDS) or reduced (in the presence of 2% β-mercaptoethanol or 100 mM dithiothreitol) and native mode, depending on the nature of the samples under analysis. The gels were stained with Coomassie Brilliant Blue R or immunoblotted.

LPS Urea/SDS-PAGE—Gel electrophoresis of LPS was performed as described by Tsai and Frasch (1982), using *Salmonella* wild-type LPS and *Salmonella* Re 595 LPS as standards (Sigma). Polyacrylamide gel concentration was either 14% or 8–15% gradient. Running and stacking gel buffers contained both 4 M urea and 0.1% SDS. Following separation, gels were stained with silver or immunoblotted with purified IgA_m, IgA_d, or IgA_p.

Immunoblotting

IgA Heavy and Light Chain—Nonspecific binding sites on nitrocellulose or polyvinylidine difluoride membranes (Bio-Rad) were saturated

for 1 h at room temperature by incubation in a blocking buffer made of PBS, 10% BSA (Fluka), and 0.05% Tween 20 (Sigma). The membrane was probed for 2 h at room temperature with either biotinylated goat anti-mouse IgA heavy chain antibody (Amersham) or with biotinylated goat anti-mouse κ chain specific antibody (Amersham), diluted 1:1,000 in PBS/0.05% Tween 20. Bound antibodies were detected using streptavidin coupled to HRP and the enhanced chemiluminescence kit from Amersham.

IgA J Chain—Nonspecific binding sites were saturated for 1 h at room temperature by incubation in a blocking buffer made of 25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.5, 5% non-fat dry milk, and 0.05% Tween 20. The membrane was probed for 2 h at room temperature with rabbit anti-human J chain serum (Hendrickson *et al.*, 1995) diluted 1:1,000 in blocking buffer for 2 h. Bound antibodies were detected with HRP-conjugated anti-rabbit immunoglobulin antibodies (Sigma) and the enhanced chemiluminescence kit from Amersham.

Human SC—Detection of the SC-IgA complexes was performed using the procedure given in Rindisbacher *et al.* (1995).

LPS—Polyvinylidine difluoride membranes were blocked with PBS, 10% BSA, 0.05% Tween 20 prior to incubation with 1 μg of IgA_d, IgAp, and sIgA for 1 h. The interaction between LPS on the membrane and various IgA forms was detected using goat anti-mouse IgA heavy chain antibody, and rabbit anti-goat HRP-conjugated IgG.

Surface Plasmon Resonance (SPR) Measurements

Measurements were performed on a home-built setup using a Kretschmann configuration (Kretschmann, 1972), as schematically shown in Fig. 1. Adsorption of molecules to, or desorption from, the surface shifts the angle of surface plasmon resonance (Knoll, 1991). For organic layers in which the thickness d is much smaller than the light wavelength, the angle shift is proportional to the change in optical thickness of the layer (which is the product $\Delta n \times \Delta d$, where the refractive index difference is $\Delta n = n_{layer} - n_{buffer}$ and Δd the change in geometrical thickness). For molecular layers with an index of refraction $n = 1.45$, the experimental angle resolution of 0.01° allowed the detection of thickness changes of 1 Å, with a time resolution of 1 measurement every 10 s.

Preparation of LPS Membranes on Gold Surfaces and IgA Binding

A supported monolayer of LPS was formed on an alkylated gold surface by exposing the support to a LPS vesicles dispersion, a procedure analogous to vesicle spreading techniques used for formation of phospholipid monolayers on alkylated surfaces (Kalb *et al.*, 1992; Terrettaz *et al.*, 1993). Glass slides with evaporated 50 nm gold film were immersed overnight in a 1 mg/ml solution of tetradecanethiol in ethanol to cover the gold with a self-assembled, tightly packed thioalkane layer (Bain *et al.*, 1989). LPS vesicle dispersions were produced by four times sonication of 1 mg of LPS in 50 μl of 25 mM phosphate buffer, pH 7.0, in a bath-type sonicator (Sonorex PK, Modell 102p) for 3 min. The clear vesicle dispersion obtained was diluted to a final LPS concentration of 1 mg/ml. 400 μl of this dispersion were placed into the cuvette, and LPS adsorption to the surface was monitored with SPR. When a stable signal had been attained (usually after 1 to 1.5 h), indicating that LPS layer formation was complete, excess LPS was removed by diluting 1:1 (v:v) with 25 mM phosphate buffer 10 times, while continuously maintaining the LPS layer covered with buffer. The SPR angle obtained after this rinsing procedure was used as a packing measure of the LPS layer.

To measure IgA_d binding to these supported LPS monolayers, first the phosphate buffer was exchanged by PBS by diluting 10 times 1:1 with PBS, then 200 μl of PBS were removed, leaving 200 μl in the cuvette to cover the LPS monolayer. At the beginning of the IgA_d concentration series, the baseline was recorded. Then, 200 μl of IgA_d solution of two times the desired concentration was injected, mixed with the solution in the cuvette, and the binding was recorded. At low IgA_d concentrations, intermediate injections were performed to account for adsorption of IgA_d to the cuvette walls, by replacing 200 μl of the solution in the cuvette with 200 μl of the simple IgA_d concentration. When binding at the given concentration was complete, a rinsing step was done by diluting 1:3 (v:v) with PBS 2 to 5 times, depending on the previous IgA_d concentration in the cuvette. From the SPR angle after the rinse step, the base line was subtracted to give the angle shift corresponding to the amount of bound IgA_d at the respective concentration. The binding at the next higher IgA_d concentration was then measured.

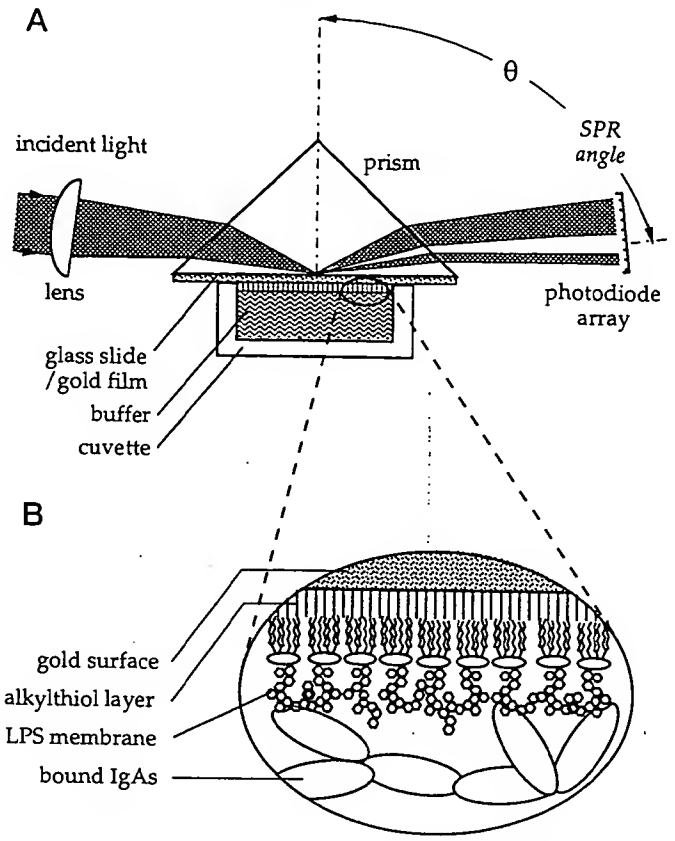


FIG. 1. **Setup for surface plasmon resonance measurements of IgA binding to LPS membranes (not drawn to scale).** *A*, optical configuration, a 60° SF10 glass prism is assembled via an index-matching oil to a glass slide onto which a 50 nm gold film has been evaporated. This assembly is pressed against a Teflon half-cuvette with the gold surface exposed to the solution in the cuvette. A parallel, monochromatic (633 nm) *p*-polarized light beam is focused by a cylindrical lens onto the gold film at the prism base, yielding a defined range of incidence angles. The resonance curve, representing the intensity of the reflected light as a function of angle, is recorded by a photodiode array connected to a PC. The angle of surface plasmon resonance (SPR angle) is marked by a minimum of intensity in the reflected light beam. *B*, model of molecular layer assemblies at the gold surface. From *top* to *bottom*, in a first step, a tetradecanethiol layer is self-assembled onto the bare gold to render the surface hydrophobic. In a second step, this surface is exposed to a suspension of LPS vesicles in buffer, generating a LPS monolayer on top of the alkylthiol layer. Finally, IgA or sIgA, respectively, is injected into the cuvette, and the antibody binding to the LPS surface is monitored.

Microcalorimetry

Calorimetric measurements (Wiseman *et al.*, 1989) were carried out using an MCS isothermal titration microcalorimeter (MicroCal Inc., Northampton, MA). Due to the amphiphilic nature of LPS, measurements were performed in 50 mM octyl-β-D-glucopyranoside (Fluka) dissolved in PBS. The 1.36-ml cell was filled with a 1 mg/ml IgA solution in octyl-β-D-glucopyranoside/PBS, corresponding to an antibody concentration of 2.9 μM. A solution of 1.5 mg/ml LPS in octyl-β-D-glucopyranoside/PBS was stepwise injected from a 250-μl syringe at 5-min intervals in portions of 12 μl (except the first shot, which was 1 μl), while stirring at 400 rpm. At this LPS concentration, an average of one LPS molecule is present per three detergent micelles; thus, no cross-linking by antibodies occurs. Water was used as reference and the instrument was calibrated by standard electrical pulses. Data analysis was performed using the Origin software and routines delivered by MicroCal together with the instrument. As LPS represents a mixture of species with different sugar chain lengths, an average molar mass had to be assumed. In a first step, this was set to 5,000 and the heat per injection was fitted with a single-site binding model with, as free parameters, number of ligands (N) per antibody, affinity constant K, and molar heat of binding H. From the obtained value of N, the average LPS molar mass was readjusted to give a binding stoichiometry of $n = 4$ (new molar mass: 10,400), and the fit was repeated to give new values K and H.

RESULTS

IgA Purification—IgA secreted by ZAC3 hybridoma cells was found to comprise a mixture of four different molecular forms, the apparent sizes of which were determined by electrophoretic mobility in SDS-polyacrylamide gels and by size exclusion chromatography.[2] The values obtained were 160 kDa for monomers, 340 kDa for dimers, 560–800 kDa for polymers, and >1,200 kDa for aggregates.

DEAE-Sepharose FF beads allowed a quantitative binding of IgA under low salt conditions. Using stepwise salt elution, it was observed that 90% of the IgA mixture loaded onto the column eluted at 200 mM NaCl. Residual protein was eluted with 500 mM salt (Fig. 2*A*). The presence of IgA in the 200 mM salt eluate was assessed by immunodetection. Analysis by SDS-PAGE and Coomassie Blue staining of this material revealed the presence of several minor contaminants (Fig. 3*A*, *lanes 3* and *4*). Size exclusion chromatography was consequently selected to separate the various IgA forms prepurified by passage on the anion exchanger. Two different resins and column sizes were tested. AcA 22 Ultrogel, with a fractionation range of 100–1,200 kDa, yielded several fractions with either pure dimers or polymers, but was considered unsuitable for large scale purification due to an unacceptable flow rate (2.0 cm/h). In order to overcome this problem, Sephacryl S300 HR with a fractionation range of 10–1,500 kDa was tested. While no baseline separation could be achieved between dimers and polymers (Fig. 2*B*), this procedure allowed the reproducible recovery of purified dimer in one-fourth of the total IgA-containing fractions (Fig. 3*C*). The optimal resolution was obtained by loading 10–30 mg of IgA mixture in a 10-ml sample volume, at a flow rate of 6.1 cm/h. DEAE anion-exchange chromatography was optimized for final concentration of the various purified IgA forms. It was observed that the best results were obtained using a linear salt gradient ranging from 200 to 500 mM NaCl.

The overall yield and purity of the different molecular forms obtained using the procedure based on separation with Sephacryl S-300 are reported in Table I. Provided that only 30% of the initial total IgA was IgA_d, the true yield of purified dimeric form was as high as 53.7%, a value which is considerably higher than any previously published protocols (Mestecky and Kilian (1985) and references herein). The final purity was estimated to be 90% for monomers and 99% for dimers and polymers. Similar figures were obtained with the AcA 22 Ultrogel column.[2] Minor contaminants remained associated with IgA as shown by Coomassie Blue staining of SDS-polyacrylamide gels with high protein loading (Fig. 3*C*). Immunological characterization of contaminating polypeptides in IgA fractions indicated that they are not related to α chain, κ chain, or J chain (Fig. 3*B* and Fig. *4*).

Biochemical Characterization of Purified IgA—The polypeptide content of the purified IgA forms was assayed by immunodetection using a battery of antibodies and antiserum against the α chain, the κ chain, and the J chain. Polymeric and dimeric forms contain all three chains, indicating that assembly of the protein occurred in the hybridoma cell. Fig. 4 shows the migration pattern obtained by SDS-PAGE under nonreducing (Fig. 4, *A* and *B*) and reducing (Fig. 4, *C* and *D*) conditions of independent preparations of IgA. Detection with anti α chain antibody (Fig. 4*A*) revealed the covalent nature of the association between the heavy chains in the different existing molecular forms. Detection of the IgA forms with anti-κ chain antibody indicates that partial covalent association of the light chain took place in monomers and dimers, but not in polymeric

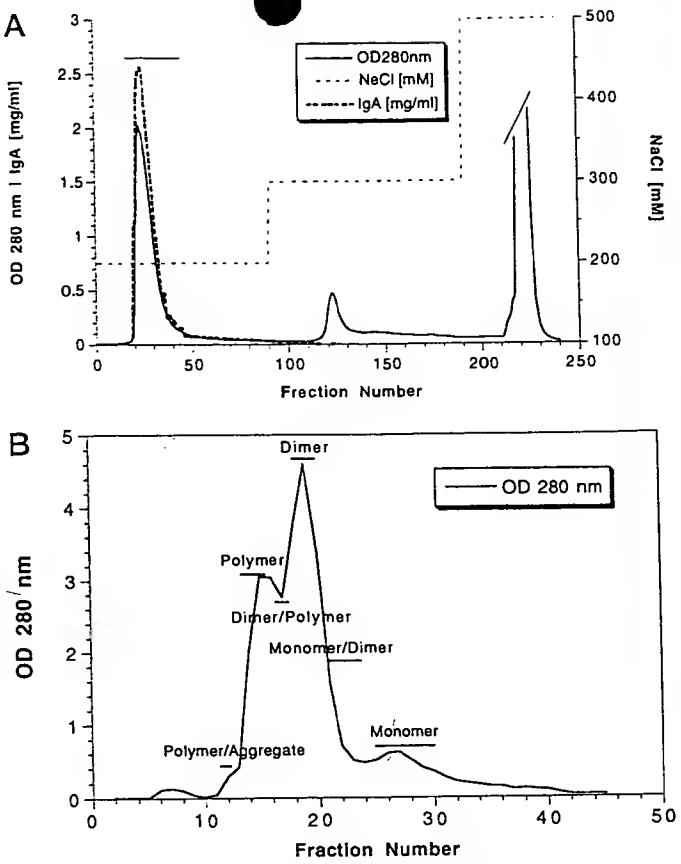


FIG. 2. **Fractionation of ZAC3 hybridoma supernatant.** *A*, elution profile of the 500-ml DEAE-Sepharose FF column run as described under "Experimental Procedures." IgA-containing fractions were identified by ELISA. *Bar* indicates the pooled fractions. *B*, monitoring of the elution profile of the Sephacryl S-300 column starting after the void volume. Fraction size was 9 ml. The different IgA forms were identified by Western blotting. IgA concentration was only measured after the fractions containing the same IgA forms were pooled according to the *bars* on the *top* of the peaks.

structures (Fig. 4*B*). Fig. 4*C* shows detection of both the α chain and the κ chain as single bands, demonstrating the absence of protein degradation during the process of purification. Immunodetection by rabbit antiserum against J chain indicates that all forms of purified IgA contain J chain, yet to a different extent (Fig. 4*D*). Under nonreducing conditions, J chain is covalently associated within IgA_d and IgA_p molecules, as shown in Fig. 4*E*.

Antigen Binding of Purified IgA with and without Secretory Component—The biological activity of the purified IgA forms was tested in a so-called "ELISA specific assay," where the binding of IgA to lipopolysaccharide antigen from the outer membrane of *V. cholerae* could be detected. Both polymers and dimers did exhibit strong, specific, and apparently similar binding to the LPS antigen, whereas IgA monomers showed no binding. Using equal amounts of protein, less binding to LPS for the IgA preparation from the AcA 22 Ultrogel column was repeatedly observed. However, since ELISA is accurate to ≤10%, the two procedures were judged to yield comparable and satisfactory levels of biologically active IgA antibody in different purified molecular forms. We next examined whether rSC might influence the binding of IgA_d and IgA_p to LPS antigen. This was undertaken by *in vitro* reconstitution of sIgA (see below), followed by determination of the antigen-antibody interaction. No significant difference was observed for IgA_d and IgA_p carrying or lacking rSC (Table II), which suggests that binding of rSC to IgA does not affect antigen recognition.

[2] E. Lüllau, unpublished data.

Reconstitution of IgA-SC Complexes in Vitro—The specificity of association of dimeric and multimeric IgA with rSC was assessed *in vitro* by combining overnight the partners in PBS buffer. As shown previously, rSC can serve as a specific ligand for IgA (Rindisbacher *et al.*, 1995) and behaves identically as human SC recovered from milk. Reconstituted complexes were loaded onto a 6% SDS-polyacrylamide gel, blotted to polyvinylidine difluoride membrane, and detected with antiserum against rSC. Fig. 5*A* shows that covalent reconstitution took place as indicated by the shift of rSC to the position of IgA_d and IgA_p molecules. Under reducing conditions, only free SC could be detected to a similar extent in every lane, indicating that SC-IgA complexes were held together through disulfide bridges. No formation of SC-IgA complex was observed with IgA_m.[3] Using densitometry scanning, we repeatedly noticed that on immunoblots the signal of free rSC was 3-fold stronger than the signal generated by the same amount of protein bound to IgA. Based on this observation, the extent of covalent association was estimated to reach approximately 80% with IgA recovered from the Sephacryl S-300 column (Fig. 5*A*). This is consistent with published values using *in vitro* reconstituted human secretory IgA (Lindh and Björk, 1976; Goto and Aki, 1984) and sIgA purified from milk samples (Mach, 1970; Weicker and Underdown, 1975).

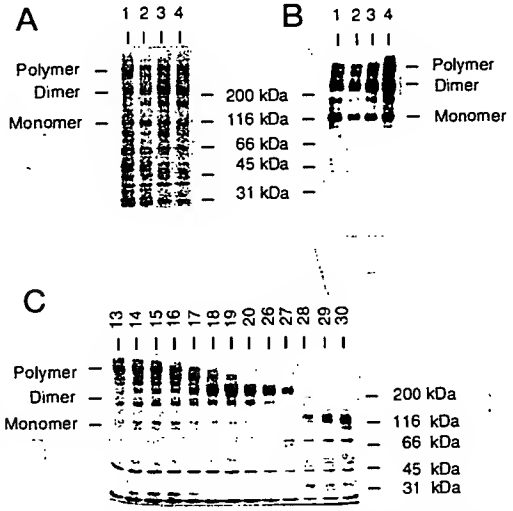


FIG. 3. **SDS-PAGE analysis of purification intermediates.** Selected samples were run under nonreducing conditions on gradient gels (4–12%). Unless otherwise indicated, the gel slots were loaded with 10 μg of protein for Coomassie stain (*A* and *C*) and 100 ng for immunodetection (*B*). *A* and *B*, starting material (*lane 1*), retentate (*lane 2*), pooled IgA-containing fractions from the 500-ml DEAE-Sepharose column (*lane 3*), pooled IgA-containing fractions from the 50-ml DEAE-Sepharose column (*lane 4*). *C*, Sephacryl S-300 fractions. *Numbers* on *top* of the figure correspond to the elution fractions in Fig. 2*B*.

For antigen binding experiments, dimeric IgA-rSC complexes were purified further by high pressure gel filtration chromatography. Increasing molar ratios of rSC to IgA_d were reacted at room temperature for 16 h, prior to separation of the sIgA from the excess of rSC on sizing columns. A typical elution profile is shown in Fig. 5*B*. The content of the peak fractions was further checked by immunodetection using antisera against rSC (Fig. 5*B*) and IgA α chain (not shown). The degree of covalent association was tested under nonreducing conditions and confirmed to be in the range of 80%. Incubation of IgA_d with rSC in molar ratios of 1:2 and 1:3 did lead to the production of a rSC-IgA_d complex with a 1:1 stoichiometry as when a 1:1 ratio of IgA_d and SC was used (Fig. 5*C*). Such purified sIgA in PBS was then quantified and used in parallel with unreconstituted IgA_d subjected to the same experimental conditions, in order to directly compare the antigen binding properties of the two IgA species.

Characterization of LPS—Analysis of *V. cholerae* LPS by SDS-PAGE revealed that it is composed of at least 6 components of different molecular weight. The smallest fraction migrated closely to the *Salmonella* Re 595 LPS standard, which

[3] B. Corthésy, unpublished data.

TABLE I
Fractionation scheme for ZAC3 cell culture harvest

	Volume	Protein	IgA	Purity	Yield
	liter	*g/liter*	*mg/liter*		%
Procedure 1					
Culture harvest	10	0.226[a]	26.4	0.12	100
Ultrafiltration cut-off 100-kDa	1.63	0.512[a]	133.3	0.26	82
DEAE-Sepharose (500 ml)	0.36	0.565[a]	500	0.88	68
DEAE-Sepharose (50 ml)	0.032	5.740[b]	5319	0.93	64
Sephacryl S-300	2.016				45
Aggregate					
Aggregate/polymer	0.126		9.5		0.5
Polymer	0.315		97.5	0.99[c]	11.6
Polymer/dimer	0.378		57.9	0.99[c]	8.3
Dimer	0.315		134.6	0.99[c]	16.3
Dimer/monomer	0.252		46.4		4.4
Monomer	0.630		19.0	0.90[c]	4.5
Procedure 2					
Culture harvest	10	0.258[a]	38	0.12	100
Ultrafiltration 1 cut-off 100-kDa	1	0.576[a]	150	0.26	39
DEAE-Sepharose (500 ml)	0.2	0.780[a]	1082	0.88	37
Ultrafiltration 2 cut-off 10-kDa	0.02				
AcA 22 Ultrogel	0.238				17
Aggregate	0.021		1.6		
Aggregate/polymer	0.035		15.5	0.99[c]	4.1
Polymer	0.021		8.2	0.99[c]	2.2
Polymer/dimer	0.028		16.9	0.99[c]	4.4
Dimer	0.035		17.1	0.99[c]	4.5
Dimer/monomer	0.028		0.9	0.90[c]	0.2
Monomer	0.070		5.9	0.90[c]	1.6

[a] Total protein determined with the Bio-Rad protein assay using IgA as standard.
[b] Total protein measured by optical density at 280 nm.
[c] Estimated by Coomassie staining of SDS-polyacrylamide gels.

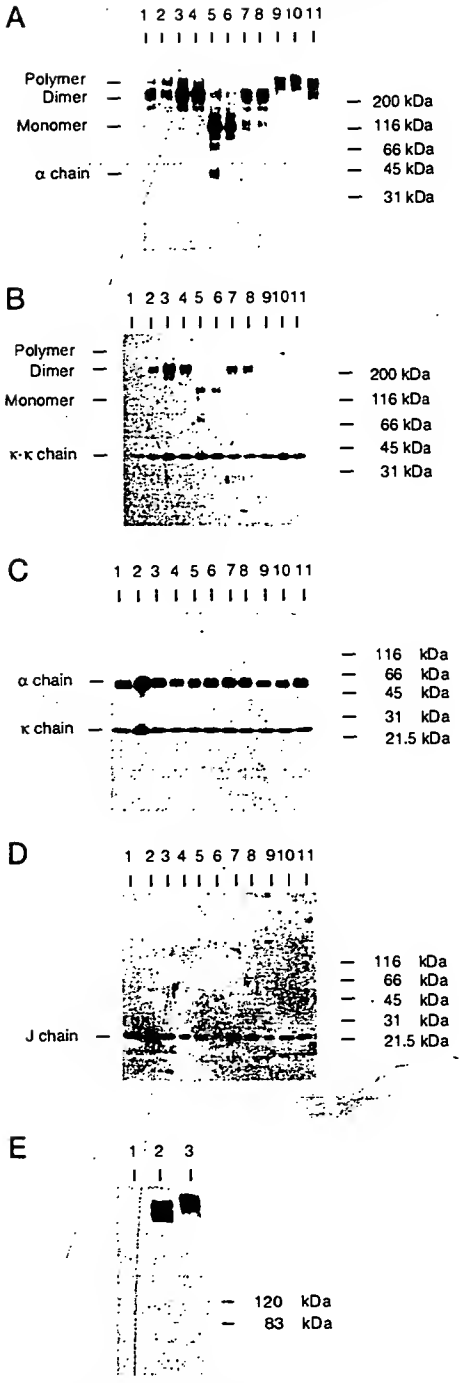


FIG. 4. **Biochemical characterization of purified IgA.** Western blot analysis of IgA recovered following procedure 1. Samples were applied to SDS-polyacrylamide gradient gels (4–12% for *A*, *B*, and *E*; 4–15% for *C* and *D*) derived from the pooled fractions recovered after the Sephacryl S-300 column and eluted from the 5-ml DEAE-Sepharose concentrating column at 250 mM in 10 mM potassium phosphate (pH 7.3), 0.02% sodium azide (KP_i). Terms in brackets indicate the storage conditions. The content of the lanes was as follows. *Lane 1*, polymer/dimer (PBS); *lane 2*, polymer/dimer (KP_i); *lane 3*, dimer (PBS); *lane 4*, dimer (KP_i); *lane 5*, monomer (PBS); *lane 6*, monomer (KP_i); *lane 7*, dimer/monomer (PBS); *lane 8*, dimer/monomer (KP_i); *lane 9*, polymer (PBS); *lane 10*, polymer (KP_i); *lane 11*, polymer/dimer (KP_i). *A*, signal obtained with 100 ng of IgA per lane using anti-mouse α chain antibody under nonreducing conditions. *B*, signal obtained with 150 ng of IgA per lane using anti-mouse κ chain antibody under nonreducing conditions. *C*, signal obtained with 100 ng of IgA per lane using anti-mouse α and κ chain antibody under reducing conditions. *D*, signal obtained with 300 ng of IgA per lane using anti-J chain antiserum under reducing conditions. *E*, signal obtained with 300 ng of IgA per lane using anti-J chain antiserum under nonreducing conditions. *Lane 1*, IgA_m; *lane 2*, IgA_d; *lane 3*, IgA_p.

TABLE II
Binding of IgA and sIgA to LPS antigen

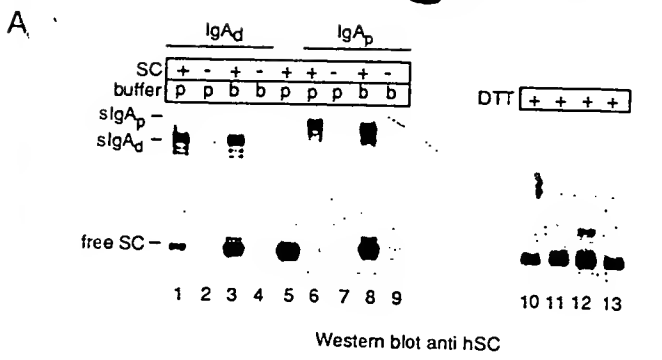
	Phosphate buffer Procedure 1		Borate buffer Procedure 2	
		% *binding*		
IgA_m	2	2	1.5	1.5
IgA_d	75.4	81.4	54.2	60.6
$sIgA_d$	81.2	83	62.6	67.4
IgA_p	96	94.7	86.7	87.3
$sIgA_p$	94	95	90	91.5

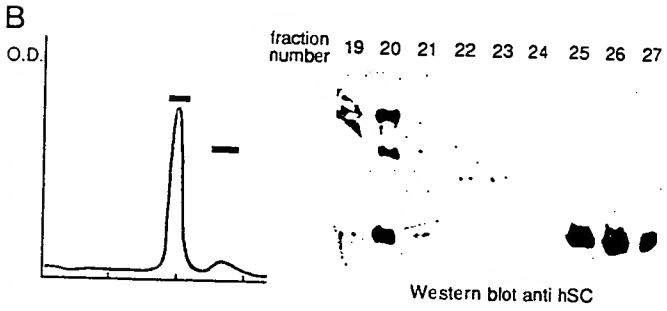
LPS is composed of lipid A and three octulusonic acid units (Brandenburg, 1993) with a molecular mass of around 2,700 Da. Thus, the molecular mass of the smallest component of *V. cholerae* LPS appears to be in the range of 3,000 to 4,000 Da. The specificity of purified IgA toward the different *V. cholerae* LPS components was assayed by blotting the gel and incubating with IgA at a concentration of 100 ng/ml and 1 μg/ml. It turned out that all LPS fractions were recognized by the antibody (Fig. 6), suggesting that the epitope is situated in the lipid A or core sugar region of LPS and not in the *O*-specific chain.

Assembly of LPS Monolayers on Alkylated Gold Surfaces—The interaction of IgAs raised against *V. cholerae* bacteria with their membrane-bound antigens was studied in a reconstituted system consisting of LPS membranes on alkylated gold supports. This arrangement mimicked LPS structure in the outer bacterial membrane and allowed quantification of antibody binding to the artificial LPS membranes by surface plasmon resonance. Film balance measurements (Ulman, 1991) showed that *V. cholerae* LPS forms stable monolayers on the air-water interface. Exposure of an alkylated gold surface to LPS-containing vesicles led to adsorption of material to this surface within 1–2 h. The total SPR angle shifts observed for all LPS membrane preparations ranged from 0.3° to 0.4°, with an average of 0.37° ± 0.07°. A refractive index of $n = 1.45$ corresponding to a 24-Å organic layer on the alkylated gold was obtained. By comparison, Fukuoka *et al.* (1994) measured a thickness of 27 Å for a monolayer of *Erwinia carotonova* rough mutant LPS. It is thus reasonable to conclude that the self-assembled structure on the alkylthiol surface corresponds to a LPS monolayer.

Binding of IgA and sIgA to Supported LPS Monolayers—To obtain binding constants of IgA_d and sIgA toward LPS monolayers, SPR measurements were performed as shown in Fig. 7. Binding affinities of IgA were determined with the antigen present in the form of a self-assembled LPS monolayer modeling a bacterial outer membrane. Because the surface could not be recycled, a new LPS monolayer had to be prepared for each experiment. This layer usually had a different optical thickness, indicating variations in LPS packing density on the surface. The SPR angle shifts for LPS membranes used for the IgA and sIgA binding experiments shown in Fig. 7 were 0.42° and 0.36°, respectively. Fig. 8 shows the angle shifts due to antibody binding using increasing antibody concentrations. Because no irrelevant dimeric IgA of comparable purity was available, values of nonspecific IgG binding to LPS monolayers were used to correct the binding data at high antibody concentrations (Fig. 8*A*), to obtain the data shown in Fig. 8*B*.

By comparing the binding curves in Fig. 8*B*, it is obvious that the secretory component does not alter the affinity of IgA_d for LPS membranes (K_A 1.0×10^8 M^{-1}). This is both evident from Fig. 8*B* comparing the concentration values for half-maximal binding in the specific binding curves (9 nM) as from the dissociation constants evaluated from Langmuir fits (both IgA and sIgA: 10 nM). Due to the higher molecular mass of sIgA with respect to IgA_d, more material adsorbs at a given concentra-





C

Molar ratio IgA/SC	IgA (μg in sIgA peak)	SC	free SC (μg in SC peak)
1.0	20.1	5.2	0.1
0.5	20.0	5.1	4.9
0.33	20.1	5.0	10.2

FIG. 5. **Analysis of *in vitro* reconstituted sIgA.** *A*, covalent reassociation of rSC with purified IgA_d and IgA_p recovered in phosphate buffer (*p*; *lanes 1* and *6*) and borate buffer (*b*; *lanes 3* and *8*). Samples were separated by SDS-PAGE under nonreducing conditions, and specific sIgA covalent complexes were detected with anti-rSC antiserum. No signal could be observed in the absence of rSC in the reassociation mixture (*lanes 2*, *4*, *7*, and *9*). rSC alone was loaded in *lane 5*. *Lanes 10–13* contain the same samples as in *lanes 1*, *3*, *6*, and *8* run under reducing conditions. *B*, molecular sieving chromatography of reconstituted IgA_d-rSC (1:2 molar ratio). *Bars* correspond to sIgA and excess of rSC, as determined by Western blot analysis of the peak fractions. Note that the intensity of free rSC in *lanes 25–27* is much higher than the intensity of free and IgA-bound rSC in *lanes 19–21*. *C*, measurement by ELISA of sIgA association as a function of the IgA_d-rSC molar ratio. A 1:1 stoichiometry is achieved in all cases.

tion, leading to a bigger angle shift. Nevertheless, if the angle shifts of IgA_d and sIgA are normalized by their respective molecular masses, one obtains a binding ratio of 0.93 (mol of sIgA/mol of IgA) at antibody concentrations of 6 nM up to 590 and 570 nM for IgA_d and sIgA, respectively. This points to steric effects at the surface, limiting the adsorbed mass density.

IgA Binding to Solubilized LPS—Fig. 9 shows an isothermal calorimetric titration of IgA_d with detergent-solubilized LPS (see "Experimental Procedures") which served to determine the binding constant of one IgA binding site to one LPS antigen (single-site binding constant). From the binding isotherm, an affinity constant of $6.6 \times 10^5\ M^{-1}$ is deduced by fitting the data to a single-site binding model (Wiseman *et al.*, 1989). This is about 200 times lower than the association constant of IgAs toward LPS monolayers. As an exact average molar mass for LPS is not known, the binding stoichiometry has been set to 4 LPS molecules per IgA_d molecule. This is justified by the nearly 100% activity of the IgA preparations (Table II) and by the fact that IgA binds to all fractions of the LPS, including molecular species composed only of lipid A and some core sugars (Fig. 6).

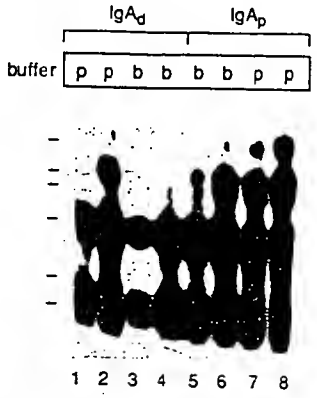


FIG. 6. **Characterization of LPS.** LPS was separated by urea/SDS-PAGE and transferred onto nitrocellulose membrane. 100 ng/ml (*lanes 1*, *3*, *5*, and *7*) and 1 mg/ml (*lanes 2*, *4*, *6*, and *8*) of purified IgA_d and IgA_p recovered in phosphate buffer (*p*) and borate buffer (*b*) were incubated with individual nitrocellulose stripes containing LPS. IgA-LPS interaction was determined using an anti-α chain antibody. The *minus sign* on the *left* indicates the six different forms of LPS bound by the antibody.

In addition, the molar heat of binding was found to be −1.9 kcal/mol.

DISCUSSION

Among the human immunoglobulins, IgA is one of the most difficult to purify. Indeed, IgA heterogeneity with respect to molecular weight is known for IgA deriving from myeloma cells and for sIgA from colostrum. Procedures developed for monomeric IgA deriving from natural sources such as human serum (Heremans, 1974; Mestecky and Kilian, 1985) are complicated processes most frequently yielding only small quantities of purified IgA. Higher molecular IgA forms are found in human myeloma cultures and in external secretions. Colostrum and early milk represent the most convenient source of human sIgA. No simple purification procedure for IgA produced by hybridoma cell culture techniques, which allows the purification and separation of different molecular forms of IgA in large quantities, has been reported so far. We have developed a simple purification scheme, based on classical chromatographic methods leading to pure and biologically active IgA_d and IgA_p.

Since IgA was produced in protein-free culture medium, the problems associated with purification due to the presence of serum IgA were abolished. As an alternative to ammonium sulfate precipitation (Mestecky and Kilian, 1985), which presents the disadvantage of exposing the samples to salts that interfere in ELISA and protein determination,[2] ultrafiltration was found to be a valuable method, allowing to concentrate IgA 10-fold with a yield of 80%. We indeed observed that a 100-kDa molecular mass cut-off did not only reduce the loading volume for the following chromatography step, but also the amount of protein (contaminants and IgA_m) in the IgA-containing fraction by about 60%. Further enrichment in IgA by a factor of 3.4 was achieved using ion exchange chromatography on DEAE-Sepharose FF resin. 80 to 90% of the IgA loaded could be eluted stepwise as a single fraction with 200 mM salt. This choice of a DEAE-type resin has been applied to the purification of human IgA (Mestecky and Kilian, 1985) and mouse IgA (Lee *et al.*, 1994). This may suggest a general use for DEAE-based ion-exchange chromatography in IgA purification or concentration. Subsequently, IgA mono-, di-, and polymers were separated in a single chromatography step. This separation required a 200-cm-long Sephacryl S-300 column (maximum load 25–30 mg of protein) and a long run (6.1 cm/h), but avoided the repetitive runs using AcA 22 Ultrogel columns reported by Vaerman (1995) for IgA from human myeloma cells.

The biochemical characterization of the different forms of

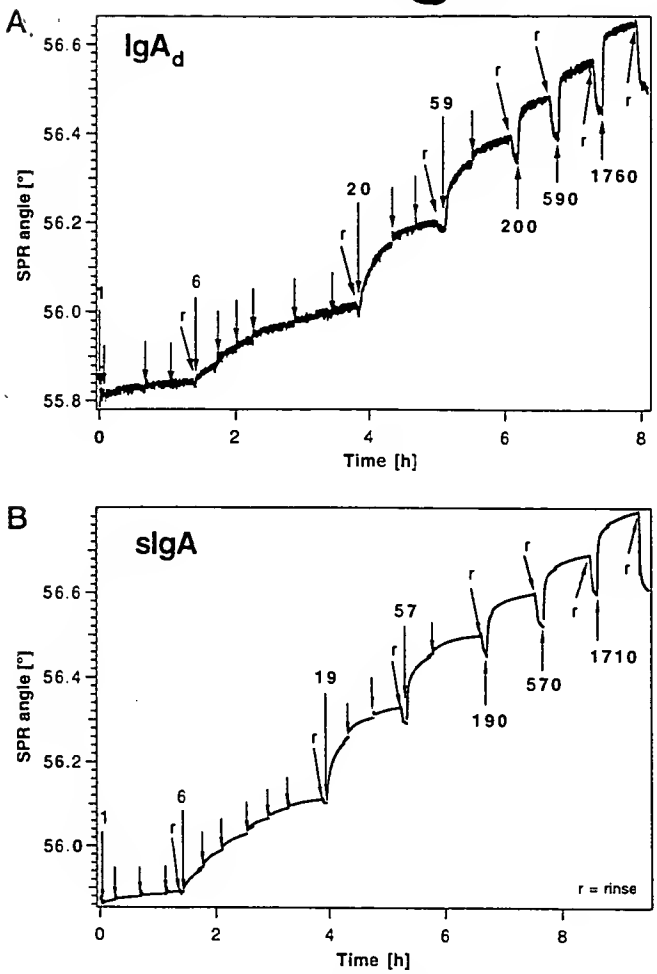


FIG. 7. **Binding of IgA_d and $sIgA_d$ to LPS monolayers on alkylated gold surfaces presented as the SPR angle *versus* time.** *A*, increasing amounts of IgA_d injected into the cuvette, expressed as final concentrations in nM (*labeled arrows*). Possible binding of antibodies to the walls of the cuvette makes intermediate reinjections at 1, 6, 20, and 59 nM antibody concentration necessary before proceeding to the next higher antibody concentration (*unlabeled arrows*). After binding at a given antibody concentration has reached saturation, the original refractive index of PBS buffer is restored by a rinse step (*r*). This leads to an angle shift when higher concentrated antibody solution in the cuvette is replaced by buffer. The difference between SPR angle values at a given IgA_d concentration (after rinse) and at the very beginning of the experiment gives the angle shift $\Delta\theta$ for this antibody concentration. *B*, same binding experiment as in *A* performed with reconstituted purified sIgA.

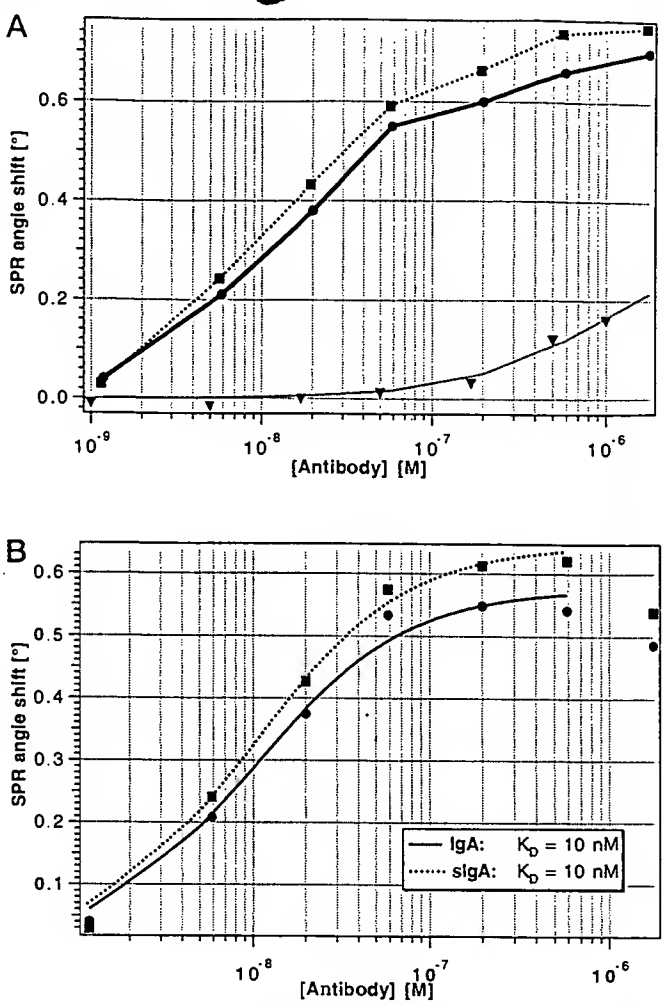


FIG. 8. **Binding curves of IgA_d, $sIgA_d$, and nonspecific IgG antibody to LPS monolayer surfaces.** The *curves* represent the SPR angle shifts deduced from the experiments shown in Fig. 7 *versus* the antibody concentration. *A*, binding of two different types of specific IgA antibodies and nonspecific IgG antibody as control was measured: specific IgA_d (●), reconstituted specific sIgA (■), and nonspecific IgG (▼). For IgA_d and sIgA, lines are connecting the measured points. Nonspecific IgG binding values were interpolated by a Langmuir adsorption isotherm fitted to the data points (*broken line*). *B*, amount of antibody specifically bound to the LPS membrane *versus* concentration, calculated as the difference between total antibody binding and binding of nonspecific IgG, for IgA_d (●) and sIgA (■). Lines are Langmuir fits to the data sets, yielding the respective dissociation constants ($K_D = 1/K_A$). It can be seen from the graph that the slopes of the binding curves are slightly steeper than predicted by the simple Langmuir adsorption model.

IgA showed that polymeric and dimeric forms contained all three α, κ, and J chain polypeptides (Fig. 4, *A–E*). Two different forms of mouse IgA exist (Vaerman, 1972): one in which light chains are disulfide-linked to each other ($IgA_{L\text{-}L}$) and the second where light chains are linked to heavy chains ($IgA_{H\text{-}L}$). In ZAC3 hybridoma, partial covalent association of the light chain to heavy chain could be detected in monomers and dimers but not in polymers (Fig. 4*B*). Under nonreducing conditions, the presence of J chain could be detected in dimeric and polymeric IgA forms only (Fig. 4*E*). However, under reducing conditions, the monomer fraction also showed a weak J chain signal (Fig. 4*D*), most likely due to traces of IgA_d (Fig. 3*C*), as reported by Weicker and Underdown (1975). Indeed, IgA_m usually does not contain J chain (Koshland, 1985; Kerr, 1990).

Binding experiments of the different purified IgA forms to an outer membrane LPS antigen from *V. cholerae* revealed that IgA monomers did not associate with LPS whereas dimers and polymers did. As published by Ishizaka *et al.* (1965), the polymeric nature of secretory IgA provides an increase in overall avidity for antigen and also an enhanced ability to cross-link multiple particles. This may explain why a dimer, or a polymeric form, is needed for antigen binding. This implies that the polymeric structure is essential for IgA to perform its function in mucosal secretions (Underdown and Schiff, 1986).

Purified IgA_d and IgA_p bind to recombinant secretory component (rSC), whereas IgA_m does not reassociate with rSC *in vitro*. Furthermore, rSC and IgA dimers or polymers covalently reassociate, reflecting a biochemical behavior similar to, if not identical with, sIgA found in mucosal and glandular secretions (Fig. 5*A*). In addition, the experiments based on size exclusion chromatography of rSC-IgA complexes show that even with an excess of rSC, one rSC molecule associates with one IgA dimer. This indicates that both partners can recognize each other with a defined intrinsic stoichiometry, rather than resulting from the nonspecific interaction of partners in solution (Fig. 5, *B* and *C*). The degree of specific reassociation between overexpressed

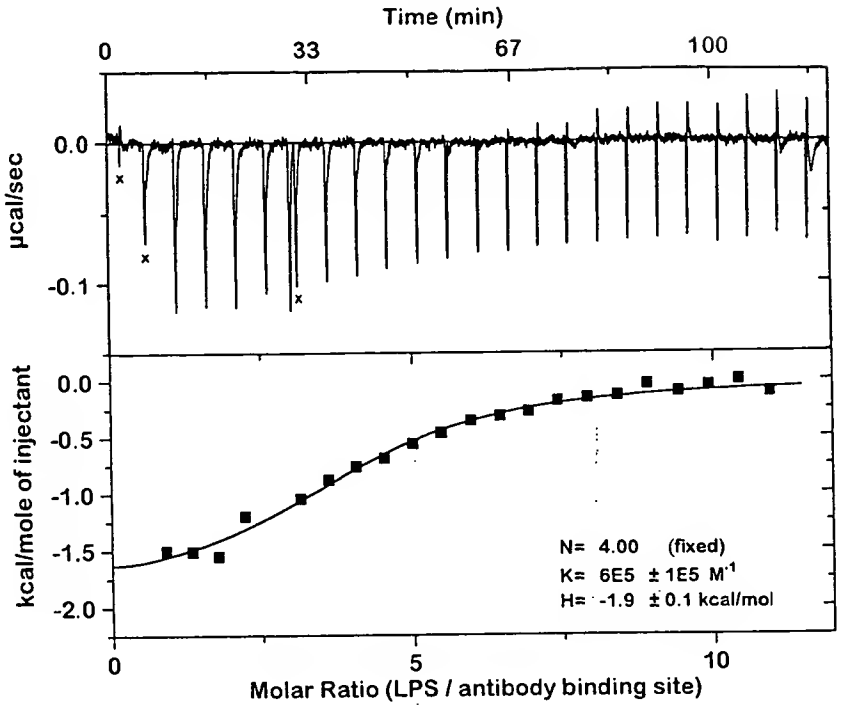


FIG. 9. **Calorimetric measurement of IgA binding to detergent-solubilized LPS.** The *upper panel* shows the differential heat during a titration of IgA_d with increasing LPS ligand. The *lower panel* shows the integrated heat per injection *versus* the molar ratio LPS:total antibody binding sites (■), along with a fit of these data by a single-site binding model (——). Free parameters of the fit were the affinity constant K and the molar heat of binding H. All injection peaks in the *upper panel* were considered for data fitting, with the exception of those marked with a *cross*.

and highly purified proteins enabled the comparative antigen binding studies described in this work.

The IgA_d binding constant to LPS membranes determined in this study (K_A 1.0×10^8 M^{-1}) has the same order of magnitude to those commonly measured for IgGs directed against peptides in solution (K_A 10^7–10^9 M^{-1}, Schwarz *et al.* (1995)) or immobilized on surfaces (K_A 10^8 M^{-1}, van den Heuvel *et al.* (1993) and Duschl *et al.* (1996)). Furthermore, the binding constant of IgA_d to LPS monolayers has been found to be two orders of magnitude higher than to solubilized LPS. The latter binding constant (K_A 6.6×10^5 M^{-1}) compares to published binding constants for IgG binding to *Salmonella O*-antigenic oligosaccharides, which are in the range of 5×10^5 M^{-1} (Sigurskjold *et al.*, 1991). We conclude that this IgA_d recognizes LPS efficiently only if it is presented as a self-assembled monolayer mimicking somehow the bacterial outer membrane surface. This may be a common feature among antibodies against carbohydrates, because their single-site affinities are usually lower than those of anti-peptide antibodies (Vyas, 1991).

The fact that the slope of the binding curves is steeper than the fit with the simple Langmuir adsorption model is consistent with reduced diffusion of free IgA_d in the vicinity of bound IgA_d at the surface. This could be caused by antibody aggregation due to the increased antibody concentration near the antigenic surface, an effect which has been observed for IgGs binding to lipid monolayers (Uzgiris and Kornberg, 1983; Wright *et al.*, 1988). The tendency of IgA to aggregate has been shown for IgA in solution (see "Results"). The molar heat of binding found in the calorimetry experiments (−1.9 kcal/mol) represents only a small part of the free energy of binding (−8 kcal/mol, deduced from the binding constant), suggesting that the IgA-LPS interaction in detergent solution is entropically driven. A pronounced entropy contribution to binding has been reported for another antibody-oligosaccharide interaction and been explained by solvent displacement upon binding (Sigurskjold *et al.*, 1991).

SPR measurements showed that SC does not change the affinity of IgAs to LPS monolayers, a result which is confirmed by the data from specific ELISA measurements. This observation is of importance, because changes in the constant part of an antibody can alter its binding constant toward an antigen-presenting surface. This has been shown for IgGs of different subclasses binding to a *N*-acetylglucosamine surface (Cooper *et al.*, 1994). The association of SC with the IgA molecule might be important to protect against proteolysis or simply reflect that it is easier to cleave and resynthesize the poly-IgR receptor than to recycle it.

Acknowledgments—We are grateful to P.-A. Ruffieux for providing us with cell culture harvests, P. Pugeaud for assistance during purification, R. Hovius for help with the calorimetric measurements, M. Liley and C. Duschl for skillful advice in SPR, and Y. Hauyon for technical assistance.

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